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CD79 α /CD79 β heterodimers are expressed on pro-B cell surfaces without associated μ heavy chain

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Abstract

During B cell development, the surface expression of CD79 α /CD79 β heterodimers had been thought to begin in the pre-B cell stage where the heterodimers constitute pre-B cell receptors together with μ heavy and surrogate light chains. Thereafter, in mature B cells, CD79 α /CD79 β associates with surface Ig to form B cell antigen receptors. In this study, we revealed by using newly established mAb that CD79 β was expressed on the surface of pro-B cells which had not undergone the productive Ig gene rearrangement. Biochemical analysis showed that CD79 β on pro-B cells existed either as monomers or as disulfide-linked heterodimers with CD79 α , non-covalently associated with four unidentified membrane molecules. Our finding that CD79 β is expressed on earlier B-lineage cells than previously expected coincides with the recent study in which CD79 β -deficient mice exhibit a blockade of B cell differentiation at the pro-B cell stage. Thus, it is speculated that the CD79 β -containing complexes on pro-B cell surfaces may function to induce early B cell differentiation.

Introduction

B cells express antigen receptors (B cell receptor; BCR) which consist of surface μ heavy chain (HC), light chain (LC) and CD79 α /CD79 β heterodimers (1). The physical association of CD79 α /CD79 β heterodimers with μ HC is necessary for the efficient transport of μ HC to cell surfaces as well as for signal transduction through the BCR (2). In spite of such close relationship between CD79 α /CD79 β heterodimers and μ HC, they exhibit a distinct pattern of cytoplasmic expression during B cell differentiation. The cytoplasmic expression of CD79 α /CD79 β begins at the pro-B cell stage in which the productive Ig gene rearrangement is not yet accomplished, whereas that of μ HC is delayed until the pre-B cell stage (3,4). Since previous studies using mAb to human CD79 β (3,5) and polyclonal antibody to murine CD79 β (6) could not convincingly detect CD79 β on

pro-B cell surfaces, it had been speculated that CD79 β was expressed in the cytoplasm of pro-B cells but stayed there until μ HC was synthesized in the pre-B cell stage to form the pre-B cell receptor together with surrogate LC. Consequently, pre-B cells had been considered the earliest B-lineage cells to express cell surface CD79 α /CD79 β .

In this paper, however, we show the expression of a small but significant amount of CD79 β on pro-B cell surfaces by using newly established mAb to murine CD79 β . The biochemical analysis revealed that CD79 β either existed as monomers or constituted heterodimers with CD79 α on pro-B cell surfaces and was non-covalently associated with four membrane molecules distinct from μ HC. Possible functions of CD79 β on pro-B cell surfaces will be discussed.

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Table 1. Cell surface expression of CD79 β on B-lineage cells

Cell line	HC gene ^b	Surface expression (sMFI ^a) of			
		CD79 β	λ_5	V _{pre-B}	μ HC
63-24	G	12	18	3	0
40E1	DJ/DJ	23	10	3	0
38B9	DJ/DJ	31	32	7	0
230-238	VDJ ⁻ /VDJ ⁻	3	1	0	0
220-8	VDJ ⁻ /VDJ ⁻	9	7	2	0
NFS5.3	R	17	73	15	11
1A9	R	24	363	143	88
WEHI231	R	654	0	0	2612
Bal17	R	270	0	0	362

^aSpecific mean fluorescence intensity: MFI of staining with specific mAb - MFI of staining with control antibody.

^bGene rearrangements of Ig HC (VDJ⁻, non-productive V_HD_HJ_H rearrangement; DJ, D_HJ_H rearrangement; R, productively rearranged; G, germline).

Methods

Cells and antibodies

Murine B-lineage cell lines used in this study are listed in Table 1 and were described previously (6-8). Purified or peroxidase-labeled goat antibody to hamster IgG or mouse Ig, and peroxidase- or phycoerythrin (PE)-labeled streptavidin were purchased from Southern Biotechnology (Birmingham, AL). Red670-labeled streptavidin was from Gibco/BRL (Grand Island, NY). FITC-labeled, PE-labeled, biotinylated or purified mAb to CD19 (1D3), BP-1 (6C3), μ HC (AM/3), CD43 (S7), CD45R (RA3-6B2), V_{pre-B} (VP245), λ_5 (LM34) and the synthetic peptide of CD79 α (HM57) were described previously (4,8,9).

Establishment of mAb to CD79 β

CD79 α /CD79 β heterodimers were purified from WEHI231 B cells as described previously (3) and used for immunization of footpads of an Armenian hamster. After the last immunization, regional draining lymph nodes were excised and lymphocytes were fused with X63-Ag8.653 (3). Hybridoma supernatants were initially screened for positive reactivity in surface immunofluorescence of WEHI231 and then the specificity to CD79 α /CD79 β heterodimers was tested by immunoprecipitation using digitonin lysates of surface-labeled WEHI231. As a result, from ~200 hybridomas, mAb secreted by five hybridomas turned out reactive with the extracellular portions of CD79 α /CD79 β heterodimers. After several rounds of limiting dilutions, clones which stably produced mAb were obtained from the five candidates and two of them (designated HM79-12 and HM79-16) were further analyzed for the specificity to CD79 β (see Results).

Biochemical analysis

For immunoprecipitation of membrane proteins, cells were surface biotinylated and lysed in 1% NP-40 or digitonin lysis buffer (3). Cell lysates were incubated with Sepharose 4B coupled with goat antibody to mouse Ig or Protein G-Sepharose (Pharmacia Biotech, Tokyo, Japan) preincubated with HM79-12, HM79-16 or normal hamster IgG. After washing, the immunoprecipitates were eluted, separated by

one-dimensional SDS-PAGE or non-reducing/reducing two-dimensional SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). The biotinylated proteins were detected by incubating the membranes with peroxidase-labeled streptavidin and visualized by the ECL system (Amersham, Arlington Heights, IL). For Western blot analysis, immunoprecipitates were prepared from unlabeled cells, separated by two-dimensional SDS-PAGE and transferred onto membranes. The membranes were incubated with HM57 or HM79-16 and the bound antibodies were detected by peroxidase-labeled goat anti-mouse Ig or peroxidase-labeled goat anti-hamster Ig respectively, followed by the ECL system.

Immunofluorescence analysis

For the staining of cell lines, cells (1×10^6) were incubated with biotinylated antibodies (50 μ g/ml) for 20 min and the bound antibodies were detected with PE-labeled streptavidin (20 μ g/ml). Biotinylated normal hamster or rat IgG served as controls. For the staining of bone marrow cells derived from 4- to 6-week-old BALB/c mice, surface μ HC⁺ cells were eliminated by panning with Petri dishes coated with anti-IgM antibody and the CD45R⁺ cells were then enriched with MiniMACS (Miltenyi Biotec, Gladbach, Germany) as described previously (9). The purified cells were stained with 50 μ g/ml of FITC-labeled, PE-labeled and biotinylated mAb, and the biotinylated mAb was developed with Red670-labeled streptavidin. Biotinylated normal hamster IgG was used as a control of biotinylated HM79-12. The indirect detection of anti-CD45R mAb was described previously (9). Cells were analyzed on a FACScan or FACSort using Lysys II software (Becton Dickinson, San Jose, CA).

Results

HM79 recognizes extracellular epitopes of murine CD79 β

As described in Methods, two mAb, HM79-12 and HM79-16, were selected based upon the reactivity with the extracellular portions of CD79 α /CD79 β heterodimers. From digitonin lysates of surface-biotinylated WEHI231 B cells, anti-Ig antibody co-precipitated CD79 α (arrowhead) and CD79 β (arrows) together with μ HC and κ LC in reducing conditions (Fig. 1A, panel 1, lane 4) as described previously (6). When HM79-12 and HM79-16 were used for immunoprecipitation from the same lysates, the immunoprecipitated molecules appeared to be compatible to those by anti-Ig antibody (Fig. 1A, panel 1). However, the result was quite different when cells were lysed in harsh detergent NP-40 which is known to dissociate the non-covalent association between CD79 α /CD79 β heterodimers and μ HC. From NP-40 lysates, both HM79 mAb immunoprecipitated CD79 α and CD79 β without μ HC and κ LC in reducing conditions and 80-90 kDa of CD79 α /CD79 β heterodimers in non-reducing conditions (Fig. 1A, panels 2 and 3). In contrast, anti-Ig antibody immunoprecipitated μ HC and κ LC alone in reducing conditions and the IgM in non-reducing conditions from the same NP-40 lysates (Fig. 1A, panels 2 and 3), indicating that the specificity of HM79 was restricted to either CD79 α or CD79 β . To further determine the specificity of HM79, immunoprecipitation and Western blot analysis were performed. From

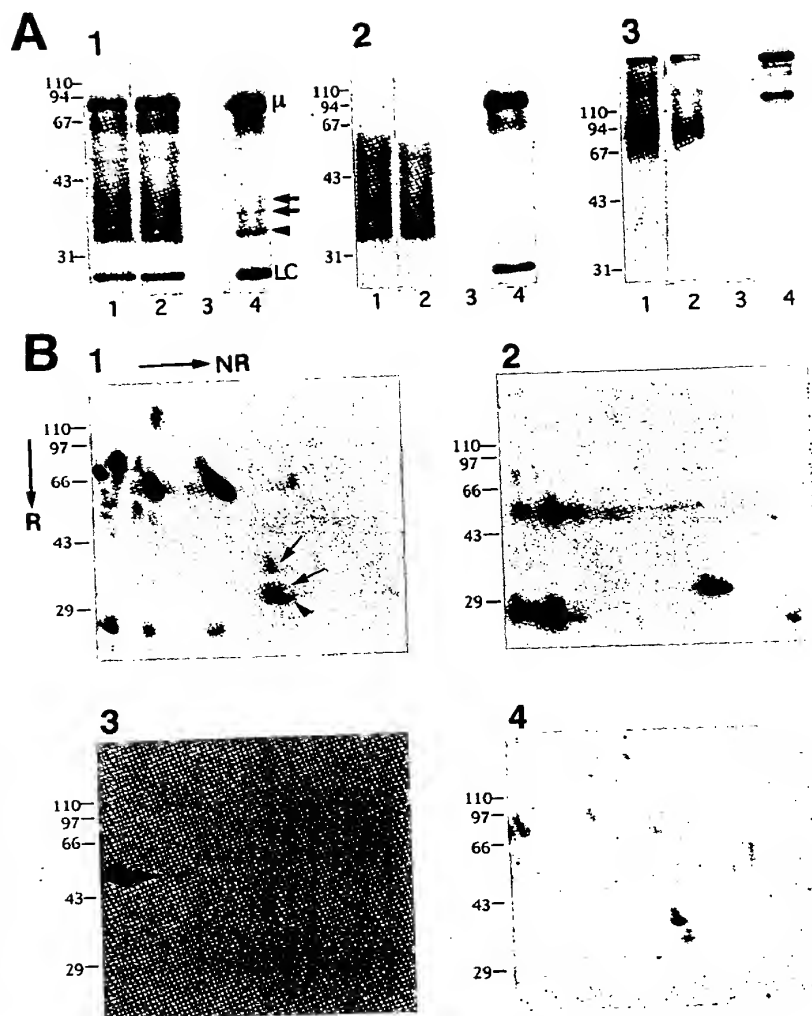


Fig. 1. HM79-12 and HM79-16 are specific for murine CD79 β . (A) WEHI231 B cells were surface biotinylated, lysed in digitonin (panel 1) or NP-40 (panels 2 and 3) lysis buffer and immunoprecipitated with HM79-12 (lane 1), HM79-16 (lane 2), normal hamster IgG (lane 3) or goat anti-mouse Ig antibody (lane 4). Immunoprecipitates were resolved by 10% SDS-PAGE under reducing (panels 1 and 2) and non-reducing (panel 3) conditions, transferred to membrane, and biotinylated proteins were visualized. Indicated in lane 4 in panel 1 are μ HC (μ), CD79 β (panel 3) conditions, transferred to membrane, and biotinylated proteins were visualized. Indicated in lane 4 in panel 1 are μ HC (μ), CD79 β (arrows), CD79 α (arrowhead) and LC. (B) Surface biotinylated (panel 1) or unlabeled (panels 2–4) spleen cells were lysed in digitonin lysis buffer and immunoprecipitated using goat antibody to mouse Ig. The immunoprecipitates were resolved by non-reducing (10%)/reducing (10%) two-dimensional SDS-PAGE and transferred to membranes. In panel 1, the biotinylated proteins were visualized with the ECL system. In panels 2–4, membranes were incubated with anti-CD79 α mAb HM57 (panel 2), HM79-12 (panel 3) or HM79-16 (panel 4), and the bound antibodies were detected by appropriate second antibodies.

digitonin lysates of surface-biotinylated spleen B cells, anti-Ig antibody precipitated two spots of differentially glycosylated CD79 β (Fig. 1B, panel 1, arrows) and one spot of CD79 α (arrowhead) together with Ig HC and LC (6). When the immunoprecipitates from unlabeled spleen B cells were subjected to Western blot analysis, the mAb to CD79 α (HM57) recognized the smaller CD79 α spot, whereas both HM79-12 and HM79-16 reacted with the larger two spots (Fig. 1B, panel 2–4), indicating that the HM79 mAb were specific to CD79 β . Off-diagonal spots between the 97 and 43 kDa size markers are murine Ig HC which are cross-reactive with

biotinylated goat anti-hamster Ig antibody. Since HM79 are reactive with viable B cells in immunofluorescence, we conclude that HM79-12 and HM79-16 recognize the extracellular epitopes of murine CD79 β .

Pro-B cell lines and normal pro-B cells in bone marrow express surface CD79 β

We next conducted the immunofluorescence analysis using HM79 with various developmental stages of B-lineage cells, and found that mature B cell Bal17, immature B cell WEHI231, and pre-B cells NFS5.3 and 1A9 (μ HC $^{+}$, V μ pre-B $^{+}$ and λ_5^{+})

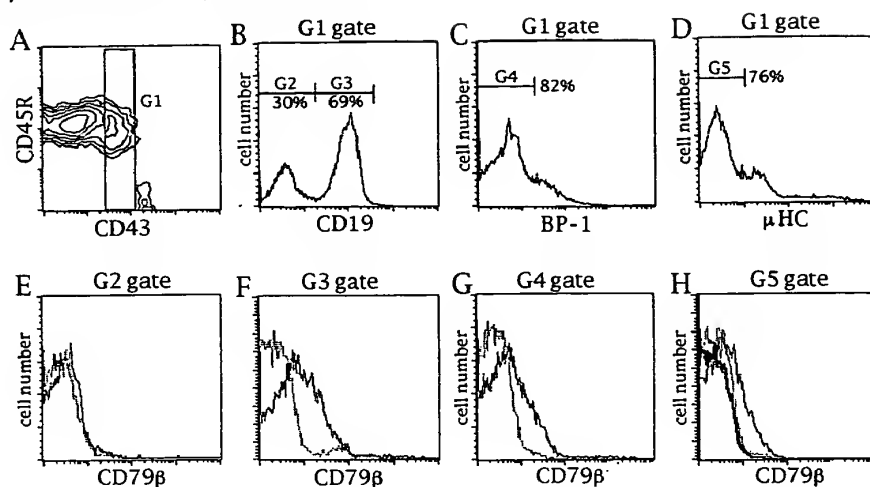


Fig. 2. CD79 β expression on pro-B cell surfaces in normal bone marrow. μ HC $^{-}$ CD45R $^{+}$ B progenitor cells were purified from bone marrow. (A) Cells were stained with mAb to CD45R and PE-labeled mAb to CD43, and the CD43 $^{+}$ G₁ gate was determined as a pro-B cell-enriched population. (B-H) Cells were stained with PE-labeled mAb to CD43, FITC-labeled mAb to either CD19 (B, E and F), BP-1 (C and G) or μ HC (D and H), and biotinylated mAb to CD79 β (HM79-12). The expression of CD19, BP-1 or μ HC in the G₁ gate was examined, and the G₂-G₅ gates were determined as described in histograms (B-D). Then, CD79 β expression in CD19 $^{-}$ CD43 $^{+}$ cells (E, G₂ gate), CD19 $^{+}$ CD43 $^{+}$ cells (F, G₃ gate), BP-1 $^{-}$ CD43 $^{+}$ cells (G, G₄ gate) and μ HC $^{-}$ CD43 $^{+}$ cells (H, G₅ gate) was shown (solid lines). Dotted lines represent the controls stained with biotinylated normal hamster IgG. In (H), binding of biotinylated HM79-12 to μ HC $^{-}$ CD43 $^{+}$ fraction (G₅ gate) in the presence of 10 times excess concentration of unlabeled HM79-12 is represented (bold line).

were all positive for surface immunofluorescence of HM79 (Table 1). In contrast to previous studies (3,5,6), however, out of five murine pro-B cells (230-238, 220-8, 63-24, 40E1 and 38B9), none of which had accomplished productive Ig HC gene rearrangements or expressed cytoplasmic μ HC (7,8), all showed a small, but significant, amount of cell surface expression of CD79 β (Table 1). All non-B lineage cell lines which we examined (P388D1, P815, BW5147 and Jurkat) were completely negative for HM79 staining (data not shown).

Since the results obtained from cell lines were unexpected, we also examined bone marrow pro-B cells for surface expression of CD79 β . Surface μ HC $^{-}$ CD45R $^{+}$ B progenitor cells were enriched from bone marrow, and stained for CD43 and CD45R. Figure 2(A) showed that the CD43 $^{+}$ CD45R $^{+}$ B progenitor population could be gated by simply setting the gate on CD43 $^{+}$ cells (G₁ gate). In this G₁ gate, 69% of cells were CD19 $^{-}$ (Fig. 2B), 82% were BP-1 $^{-}$ (Fig. 2C) and 76% were μ HC $^{-}$ (Fig. 2D), indicating that the major population of the G₁ gate had the pro-B cell phenotype (10,11). As shown in Fig. 2(E and F), the significant shift of the histograms by HM79-12 staining was observed in CD19 $^{+}$ cells (G₃ gate) but not in CD19 $^{-}$ cells (G₂ gate), which indicated that the surface expression of CD79 β was restricted to B-lineage cells in the G₁ gate. Since CD19 $^{+}$ cells in the G₁ gate had been shown to include pro-B cells and some early pre-B cells (10,11), we then examined the expression of CD79 β in the BP-1 $^{-}$ fraction of the G₁ gate (Fig. 2C, G₄ gate) which did not include pre-B cells (10). Figure 2(G) clearly showed that the BP-1 $^{-}$ pro-B cell fraction expressed CD79 β . Although the G₁ gate was contaminated with μ HC $^{+}$ cells (Fig. 2D), we could confirm that μ HC $^{-}$ cells in the G₁ gate (G₅ gate) expressed surface CD79 β (Fig. 2H, solid line). In addition, 10 times excess

amount of unlabeled anti-CD79 β mAb completely inhibited the binding of the biotinylated mAb to μ HC $^{-}$ CD43 $^{+}$ (Fig. 2H, bold line), proving the specific binding of the biotinylated mAb. From these results we conclude that bone marrow pro-B cells express surface CD79 β .

Biochemical analysis of CD79 β on pro-B cell surfaces

On mature B cell surfaces, CD79 β constitutes heterodimers with CD79 α via disulfide linkage and the heterodimers are non-covalently associated with IgM to form a stable BCR (1). Thus, it is of great interest to know how CD79 β is expressed on cell surfaces of pro-B cells without IgM. To elucidate this point, we conducted biochemical analysis using 38B9 pro-B cells which showed the highest expression of surface CD79 β in pro-B cell lines (Table 1). After cells were surface biotinylated and lysed in digitonin, CD79 β and the associated molecules were immunoprecipitated by anti-CD79 β mAb. As shown in Fig. 3(A), reducing/non-reducing two-dimensional SDS-PAGE showed that the immunoprecipitates were resolved into two off-diagonal spots and six on-diagonal spots. The off-diagonal spots appeared to be generated from the heterodimeric complex with disulfide linkage, and the Western blot analysis using anti-CD79 α mAb (HM57) and anti-CD79 β mAb (HM79-16) (Fig. 3B and C) revealed that the larger spot (Fig. 3A, arrow) was CD79 β and that the smaller one (Fig. 3A, arrowhead) was CD79 α . Two spots on the diagonal line (Fig. 3A, arrow and arrowhead) were also CD79 β and CD79 α as demonstrated in Fig. 3(C) and the longer exposure of Fig. 3(B) (not shown). Anti-CD79 β mAb precipitated four spots on the diagonal line which reacted with neither anti-CD79 α mAb nor anti-CD79 β mAb (Fig. 3A). The estimated molecular sizes of the four spots were 110, 90-60, 30 and

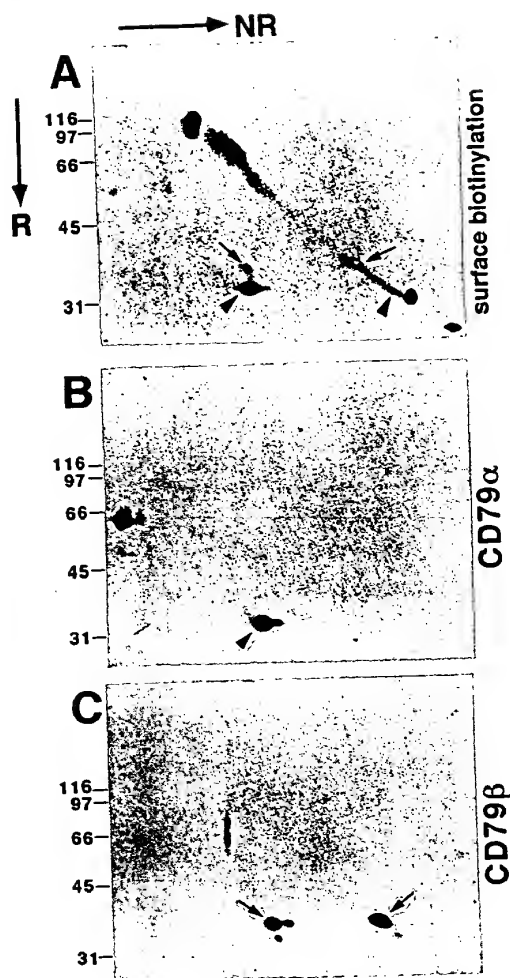


Fig. 3. Biochemical analysis of CD79 β on pro-B cells. (A) 38B9 pro-B cells were surface-biotinylated, lysed in digitonin and immunoprecipitated with anti-CD79 β mAb (HM79-16). The immunoprecipitates were resolved by non-reducing (10%)/reducing (10%) two-dimensional SDS-PAGE, transferred to Immobilon P membranes and the biotinylated molecules were visualized. Arrows and arrowheads indicate the spots reactive with anti-CD79 β and CD79 α mAb in (B) and (C) respectively. (B and C) Immunoprecipitates using anti-CD79 β mAb from digitonin lysates of unlabeled 38B9 pro-B cells were resolved by two-dimensional SDS-PAGE and transferred to membranes. The membranes were incubated with anti-CD79 α mAb HM57 (B) or anti-CD79 β mAb HM79-16 (C) and bound mAb were detected as described in the legend to Fig. 1. Arrowhead and arrows indicate the spots which were specifically reactive with each mAb.

<30 kDa, which migrated together with the front line of the gel. All migrated on the diagonal line, indicating the non-covalent association with CD79 β .

Discussion

By using novel mAb HM79 which recognize murine CD79 β , we here show the first evidence that pro-B cells expressed CD79 β on cell surfaces without associated μ HC. The specifi-

city of HM79 was biochemically confirmed using immunoprecipitation and Western blot analyses. The comparison of the results of HM79 with those of the mAb to murine CD79 α enabled us to conclude that HM79 was specific to murine CD79 β . We also established transfectants expressing the murine CD79 β gene and ensured the specific binding of HM79 to the transfectants (data not shown).

We found the unexpected surface expression of CD79 β not only on pro-B cell lines but also on normal pro-B cells in murine bone marrow. In humans, in spite of the intensive studies using monoclonal reagents, we and others could not detect the surface CD79 β on pro-B cells (3,5). Although we do not fully understand the reason for the discrepant results between mice and humans, one simple explanation could be that the regulation of CD79 β expression during B cell differentiation differs between the species. In fact, Lassoud *et al.* have biochemically shown that CD79 α and CD79 β are rapidly degraded in the endoplasmic reticulum of human pro-B cells and fail to reach the cell surface (12). Another possibility is that the antigenic epitopes on CD79 β recognized by mAb other than HM79 are accessible when CD79 β is associated with μ HC, but not accessible when expressed on pro-B cell surfaces together with molecules distinct from μ HC. A similar phenomenon has been reported in the case of mAb to surrogate LC (13).

Biochemical analysis revealed that CD79 β on 38B9 pro-B cells existed either as monomers or as heterodimers together with CD79 α and was non-covalently associated with four membrane molecules to constitute a cell surface complex on pro-B cells. We also showed that not only CD79 β but also a small amount of CD79 α was expressed as monomers on cell surfaces. The existence of monomeric CD79 α has been also reported in murine pre-B cells (14), which may suggest that immature B cells are impaired in the ability to make disulfide linkages. We showed that the anti-CD79 β mAb immunoprecipitated monomeric or heterodimeric CD79 α /CD79 β as well as four other membrane molecules from pro-B cell lysates. However, it is not clear at this moment whether all of them constitute a single cell surface complex or they constitute heterogeneous complexes. Since 38B9 pro-B cells do not express Ig HC and LC, the four molecules associated with CD79 α /CD79 β are neither HC nor LC. The candidates for the identities of these four membrane molecules would include calnexin, CD22, surrogate LC or other unidentified molecules and further studies are in progress regarding this point.

Gong *et al.* recently showed that CD79 β -deficient mice exhibited a complete block in pro-B cell development before the rearrangement of V_H to DJ_H (15). Although their finding was unexpected since CD79 β had been thought to be expressed first on cell surfaces of pre-B cells, our results shown here might give some clue to understand their finding. We revealed that CD79 β constituted novel complexes on pro-B cell surfaces. As the cross-linkage of the pre-BCR induces LC gene rearrangement in pre-B cells (16), it is possible that signals delivered through CD79 β on pro-B cells induce the recombination of V_H to DJ_H, resulting in differentiation of pro-B cells into pre-B cells. If this is the case, the disruption of the CD79 β gene would result in a block of differentiation from pro-B to pre-B cells as reported by Gong *et al.* Thus, the identities and functions of the molecules which are non-

covalently associated with CD79 β on pro-B cells are of great interest to understand early B cell development, and are now under our investigation.

Acknowledgement

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Abbreviations

BCR	B cell receptor
HC	heavy chain
LC	light chain
PE	phycoerythrin

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